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Uptake, Translocation and Metabolism of the Herbicide Molinate in Tobacco and Rice

Yar-Ning Hsieh,[1] Li-Fei Liu[2] & Yei-Shung Wang[1]*

[1] Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan
[2] Department of Agronomy, National Taiwan University, Taipei, Taiwan

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Abstract: Molinate, a selective herbicide, is used for the control of annual and perennial weeds in rice paddy fields. This study was designed to assess the basis of the selective action of molinate between a susceptible broadleaf crop, tobacco, and a resistant graminaceous plant, rice. Experiments were conducted comparing plant growth under different concentrations of molinate, determining the absorption and translocation of the herbicide in the plant and identifying the metabolites in suspension cells. Rice showed greater tolerance to molinate than tobacco. Leaves of tobacco showed retarded and distorted growth at 10 mg liter^{-1} of molinate 14 days after treatment, but rice leaves were unaffected at this concentration. Higher concentrations of molinate accumulating in the root of tobacco seedlings may inhibit root development and represent a significant factor in the herbicide's selective action. Seven and eight metabolites were found in tobacco and rice cells, respectively, with molinate sulfoxide and molinate sulfone present in both species. © 1998 SCI

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Key words: molinate; uptake; translocation; metabolism; tobacco; rice

1 INTRODUCTION

Molinate (*S*-ethyl perhydroazepine-1-carbothioate) is extensively used as a selective herbicide to control germinating broad-leaves and grassy weeds in rice paddy fields. Molinate is applied after water seeded or drilled rice is flooded and after the aquatic weed has emerged about 5–10 cm from the soil, and is at least two-thirds submerged by the water. Rates of application are from 2·2 to 3·4 kg ha^{-1}. A number of studies have been published on the fate and selectivity of molinate. Molinate controls barnyardgrass (*Echinochloa crus-galli* (L.) Beauvois) in paddy rice fields, and Chem *et al.*[1] reported that barnyardgrass absorbed more molinate than did rice. Imai and Kuwatsuka[2] found similar results, deducing that molinate only moved acropetally in rice plants, but both acropetally and basipetally in barnyardgrass when applied to the basal part of the shoots, although the molinate showed whole-plant systemicity in both plants when applied to the roots. In a study on effect of thiocarbamate herbicides on fatty acid synthesis by potato, Bolton and Harwood[3] found that thiocarbamates reduce the amount of surface lipid of plants. Evidence suggesting that thiocarbamates inhibit one or more acyl-CoA elongases is largely indirect.[4] Imai and Kuwatsuka[5–8] studied the degradation of this herbicide in soil and its metabolism by soil microorganisms. Soderquist *et al.*[9] reported that photodecomposition products of molinate were present in field water, and that volatilization of molinate from water was the primary mode of dissipation in the environment. Ross and Sava[10] determined atmospheric residues at 48 cm above the water surface, finding that the maximum molinate concentration was 48 μg m^{-3} on the day of application of 3·10 kg ha^{-1} to rice fields. In a study in rice

* To whom correspondence should be addressed.
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by Imai and Kuwatsuka,[11] 21% of the total molinate applied was detected in the soil and 1·6% in the rice plants at harvest, and of the molinate residues found in the rice plants, about 96% was in straw and 4% in the grains.

The present study was carried out to clarify the absorption and translocation of molinate in plants of tobacco, a susceptible broad-leaf, and rice. The metabolism of molinate in suspension cells tissue culture of both plants was also determined.

2 MATERIALS AND METHODS

2.1 Materials

[*Ethyl-1*-^{14}C]molinate was provided by Asahi Chemical Industry Co., Ltd, Japan, and had a specific activity of 107·3 MBq mmol^{-1} and a radiochemical purity of greater than 99%. Analytical grade molinate of greater than 99% purity was purchased from Riedel de Haen Co., Germany. Seeds of tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) and rice (*Oryza sativa* L. cv. Tainan 5) were provided by the Department of Botany and Department of Agronomy, National Taiwan University, respectively.

2.2 Seedling preparation

Seeds of tobacco were soaked in ethanol + water (75 + 25 by volume) for 1 min and sterilized with sodium hypochlorite solution (10 g liter^{-1}) for 15 min. Heavy rice seeds were selected with sodium chloride solution (50 g liter^{-1}; relative density 1·05)[12] and then surface sterilized for 2 h with sodium hypochlorite (10 g liter^{-1}), adding one drop of 'Tween'-20. After washing three times with deionized water, the tobacco seeds were germinated and grown in glass bottles containing MS agar medium[13] (50 ml) for several weeks at 25°C under 3000 lux illumination (16 h day^{-1}), and the rice seeds were germinated on a stainless steel net laid on the upper surface of liquid in glass bottles containing MS liquid medium for one week. Shoot apices of tobacco plants were excised and transplanted every three to five weeks.

2.3 Effect of molinate on growth of tobacco and rice plants

Tobacco plants (two weeks after transplantation, 5 cm in height) and rice plants (one week after germination, 7 cm in height) were transferred to MS liquid medium (complemented with sucrose and vitamins) containing 0, 1, 10 and 100 mg liter^{-1} of molinate, separately. After incubating for 14 days, gross effects or whole plant growth were noted. The experiment was performed in duplicate.

2.4 Absorption and translocation of molinate by plants

Rice seedlings and shoot apices from tobacco plants were removed and transferred to another glass bottle containing MS liquid medium (30 ml) and glass beads. The plants were fixed with glass beads. After roots had developed (seven days), [^{14}C]molinate (0·11 MBq) was added to the medium. After incubating for two and seven days, whole plants were washed with distilled water containing acetone (1·0 ml liter^{-1}) and 'Tween'-20 (1·0 ml liter^{-1}), fixed on a glass plate and then covered with X-ray film (20 × 25 cm, Kodak X-Omat AR) at − 20°C for two months for autoradiography. The plant materials were then subjected to wet combustion and the radioactivity was quantified. The test was performed in duplicate. Detailed methods are described elsewhere.[14]

For a thorough understanding of the relationship between molinate concentration and plant absorption and translocation, an experiment was also performed by adding 2·5, 5·0, 7·5 or 10·0 mg liter^{-1} of [^{14}C]molinate to the medium and determining the radioactivity in the shoot and root after incubation for two days. Each test was performed in duplicate.

2.5 Suspension cultures of tobacco leaf cells and rice embryogenic cells

For callus preparation, whole tobacco leaves from beneath the shoot apices were sliced into pieces of 1–2 cm in length, and placed on MS agar medium containing 2-(1-naphthyl)acetic acid (NAA; 1·0 mg liter^{-1}) and 6-benzylaminopurine (BAP; 0·1 mg liter^{-1}) for callus induction. Induced callus was suspended and dispersed in a MS liquid medium containing NAA,(0·5 mg liter^{-1}) and BAP, (0·1 mg liter^{-1}). The cultures were shaken at 100 rev min^{-1} at 25°C. Homogeneous suspensions were obtained by filtering with a 1-mm filter net. Subculture was performed every two weeks.

Rice embryogenic calli obtained from cultured immature embryos of rice seed were transplanted and cultured in a 250-ml flask containing MS liquid medium (50 ml) supplemented with 2,4-D (10 μM) on a shaker (100 rev min^{-1}). Homogeneous suspensions were obtained by filtering with a 1-mm filter net. Subculture was performed every seven days.

2.6 Metabolism of molinate in tobacco and rice cell suspensions

Metobolism of molinate in tobacco and rice was examined by culturing the cells in medium containing

[^{14}C]molinate (10 mg liter^{-1}). In five 50 ml-flasks, [^{14}C]molinate solution (0·037 M Bq) in ethanol was added to the suspension (10 ml; approximate cell density 4×10^5 ml^{-1}) in each flask to give 10 mg liter^{-1} concentration of molinate. The cells were shaken at 25°C under 3000 lux illumination and 50 rev min^{-1}. After incubating for 3, 6, 12, 24 and 48 h, one flask was taken and the suspension was centrifuged (1000*g*, Sigma 2K-15 12139 rotor) to sediment the cells. The cells were collected, washed with MS liquid medium (5 ml) and again centrifuges. The percentage of radioactivity remaining in the culture medium was determined. Collected cells were added to 70% acetone (10 ml), homogenized and extracted three times. The extracts were combined, diluted with water and then extracted with hexane three times. The procedure for determining molinate and its metabolites is shown in Fig. 1, a method used previously by Imai and Kuwatsuka.[2] The radioactivity of the residues was determined by the wet combustion method.[14] Metabolites were analyzed by co-chromatography with authentic standards. Hexane and ether extracts were concentrated and co-chromatographed. Polar conjugates were hydrolysed with hydrochloric acid (1 M) at 70°C for 2 h.

The metabolites molinate sulfoxide [1-ethylsulfinylcarbonyl perhydroazepine] and molinate sulfone [1-ethylsulfonylcarbonyl perhydroazepine] were prepared by the method of Soderquist *et al.*[9] and identified by GC-MS and FT-IR (Bio-red model FTS-7). GC-MS analysis was performed by using Shimadzu GC-14A interfaced to a QP1100EX mass detector equipped with a Supelco PTE 5 fused silica capillary column (30 m × 0·25 mm ID, film thickness 0·25 μm). Helium was used as the carrier gas with a flow rate of 3 ml min^{-1} with split ratio 1 : 50. The injection port temperature was 200°C; interface was 250°C; oven temperature was held at 100°C for 5 min, then programmed to 250°C at 10°C min^{-1}, and maintained at the final temperature of 250°C for 20 min. Following identification by GC-MS, the retention times were found to be 18·9 min (molinate sulfoxide) and 16·9 min (molinate sulfone), both compounds displaying base peak *m/e* 126 ($C_6H_{12}NCO$).

Thin layer chromatography was performed on silica gel 60 F_{254} (Merck 5715) using hexane + acetone (1 + 1 by volume) as developing solvent and metabolites were identified at 254 nm with a UV lamp. The R_f values of molinate, molinate sulfoxide and molinate sulfone were 0·90, 0·55 and 0·83, respectively.

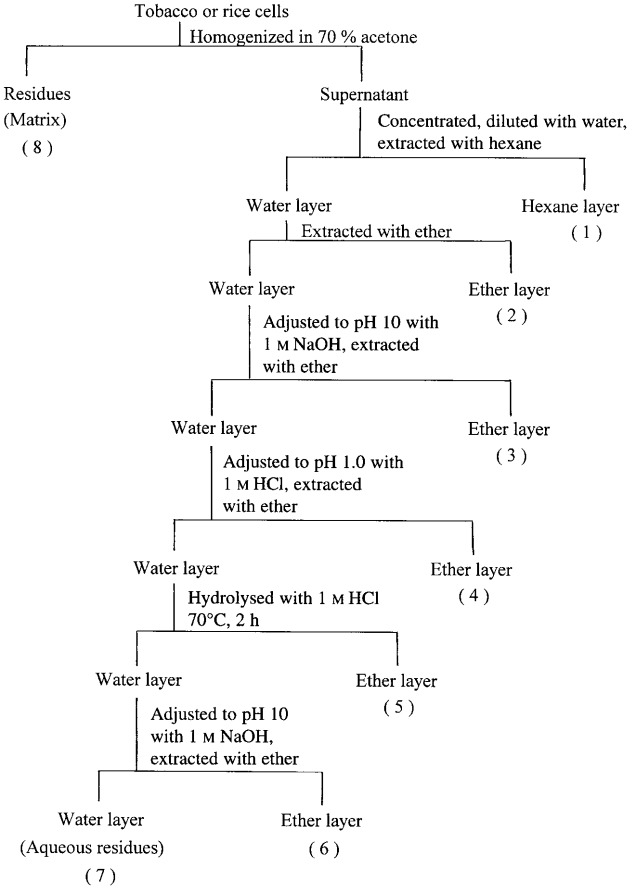

Fig. 1. Extraction of molinate and its metabolites from tobacco and rice cells.

3 RESULTS AND DISCUSSION

Effects of different concentrations of molinate on the growth of tobacco and rice plants were compared after incubation for 14 days. Growth of tobacco shoots was not affected by 1 mg liter^{-1} of molinate, but leaf distortion and growth inhibition were found at 10 mg liter^{-1}. At a concentration of 100 mg liter^{-1}, the older leaves showed necrosis and a brownish colour. Growth of tobacco roots was slightly inhibited by 1 mg liter^{-1} of molinate, and when the concentration exceed 10 mg liter^{-1}, root development was greatly inhibited. The growth of rice plants was inhibited and the leaves become a brownish colour at a concentration of 100 mg liter^{-1}. Rice plants showed more tolerance to molinate than tobacco. The lengths of shoots and roots of both species after treatment with different concentrations of molinate are shown in Table 1.

Autoradiograms (not shown) and tissue combustion of [^{14}C]molinate-treated plants indicated that, in tobacco, more [^{14}C] derived from molinate remained in the root than translocated to the shoot after two days incubation, but most of the radioactivity was translocated to the shoot at seven days, when expressed on a dpm g^{-1} basis. By contrast, in rice radioactivity translocated more rapidly to the shoot at two days after incubation. Table 2 shows the plant distribution of [^{14}C] derived from molinate. No obvious difference was found for total [^{14}C] absorption between the two species; about 0·59% (0·17% in root and 0·42% in shoot) and 0·55% (0·2% in root and 0·35% in shoot) of the total activity being absorbed by tobacco and rice,

TABLE 1
Length of Tobacco and Rice Shoots and Roots after Treatment with Different Concentrations of Molinate

Concentration of molinate (mg liter^{-1})	*Organ length (cm) (±SD)*			
	Tobacco		*Rice*	
	Shoot	*Root*	*Shoot*	*Root*
Control	4·03 (±0·40)	6·38 (±0·26)	17·00 (±0·90)	5·27 (±0·25)
1	5·03 (±0·20)	5·44 (±0·37)	19·01 (±1·22)	6·02 (±0·95)
10	3·39 (±0·23)	2·20 (±1·35)	18·44 (±0·14)	5·54 (±0·27)
100	1·68 (±0·06)	0	6·80 (±0·80)	4·06 (±0·40)

respectively, after incubation for seven days. Chem *et al.*[1] and Imai and Kuwatsuka[2] had noted that *E. crus-galli* absorbed more molinate and rice absorbed relatively less molinate and therefore this may be the reason for molinate's selectivity. The higher level of radioactivity in the roots of tobacco compared with those of rice may indicate that a critical level of molinate or its metabolites in tobacco roots may be a factor in the selective activity of the herbicide. Figure 2 clarifies this point and shows the relationship between [^{14}C]molinate concentration and accumulation by tobacco and rice plants when incubated for two days. The results showed that the higher the concentration of molinate present in culture solution, the more molinate was absorbed by both species. However, most of the molinate absorbed was translocated to the shoot in rice plants and only a small amount remained in root, regardless of the concentration. On the other hand, most of molinate absorbed by tobacco accumulated in the root, only a small amount being translocated to the shoot.

After exposing cell suspensions to [^{14}C]molinate for two days, the herbicide and its metabolites were determined. Table 3 lists the percentages of radioactivity distribution in different extraction fractions after different incubation periods. In rice cells, more than 60% of the radioactivity derived from [^{14}C] molinate was found in the aqueous residue fraction after 48 h, implying conversion to polar metabolites. Rice cells were able to metabolize molinate to polar metabolites more rapidly than tobacco cells. The relatively non-polar metabolites (hexane fraction) accounted for 15–30% in rice cells and were lower than that in tobacco cells. After acid hydro-

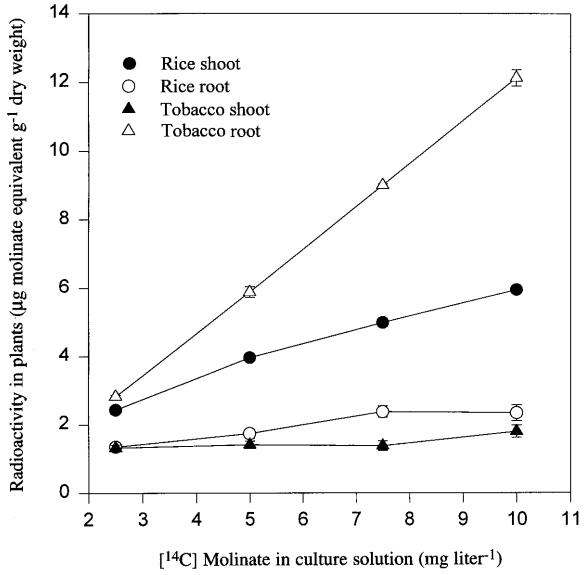

Fig. 2. Relationship between [^{14}C]molinate concentration and accumulation by tobacco and rice plants after two days.

TABLE 2
Absorption and Translocation of Radioactivity Derived from [^{14}C]molinate in Tobacco and Rice Plants

	Incubation (days)	*Tobacco*		*Rice*	
		Root	*Shoot*	*Root*	*Shoot*
Radioactivity	2	2664 (±51)	2758 (±37)	3605 (±33)	3912 (±32)
in plant (dpm)	7	11 040 (±229)	28 026 (±186)	12 950 (±59)	23 364 (±147)
Plant weight	2	0·01 (±0·000)	0·07 (±0·007)	0·07 (±0·007)	0·03 (±0·000)
(dry, g)	7	0·02 (±0·000)	0·18 (±0·007)	0·05 (±0·000)	0·06 (±0·007)
dpm g^{-1}	2	266 400 (±5100)	39 400 (±4000)	51 500 (±5200)	130 400 (±1100)
	7	552 000 (±11 400)	155 700 (±6100)	259 000 (±1200)	339 400 (±46 700)
^{14}C	2	0·04 (±0·001)	0·04 (±0·001)	0·05 (±0·001)	0·06 (±0·000)
absorbed (%)	7	0·17 (±0·003)	0·42 (±0·003)	0·20 (±0·001)	0·35 (±0·002)

TABLE 3
Distribution of Radioactivity in Different Extract Fractions when Tobacco and Rice Suspension Cells Were Incubated in [^{14}C]Molinate for Different Periods

	Radioactivity recovered (%)								
	Supernatant following extraction in 70% acetone					*After hydrolysis*			
			Ether extract			*Ether extract*			
Incubation time (*h*)	*Matrix residues* (*8*)[a]	*Hexane extract* (*1*)	*Neutral* (*2*)	*Basic* (*3*)	*Acidic* (*4*)	*Acidic* (*5*)	*Basic* (*6*)	*Aqueous residue* (*7*)	*Total radioactivity* (*dpm*)
Tobacco									
3	0·3	45·1	6·4	5·1	2·5	1·2	0·8	38·5	174 200
6	0·2	27·9	9·0	5·9	3·3	1·7	1·0	51·0	157 400
12	0·3	23·4	9·6	6·0	3·4	1·7	1·6	53·9	140 300
24	0·1	40·0	4·8	3·0	2·0	2·0	0·8	47·2	220 700
48	0·2	29·2	7·0	5·6	2·6	1·4	1·3	52·6	181 100
Rice									
3	0·2	26·8	20·7	7·4	2·7	1·0	1·5	39·6	155 500
6	0·2	30·4	18·0	4·5	2·3	0·9	0·8	42·9	179 600
12	0·2	25·4	14·1	4·2	2·4	1·1	1·3	51·2	173 600
24	0·4	20·4	10·5	4·2	3·2	1·1	1·7	58·4	148 200
48	0·2	15·0	10·4	7·9	3·6	1·2	1·6	60·1	184 700

[a] The number corresponds to the fractions in Fig. 1.

lysis, ^{14}C-compounds in the ether extracts showed no significant different between tobacco and rice cell, the sum of residues in the acidic and basic ether extracts totalling 1·7 to 3·3% of total radioactivity found in the cell. These results imply that only small amounts of [^{14}C]molinate were conjugated as glycosides or other complexes.

When the 70% acetone extract was separated by thin layer chromatography, in addition to the parent molinate, eight (in tobacco) and 10 (in rice) spots were observed on chromatographic plates. Most of these metabolites were found in cells of both species, such as molinate sulfone, (R_f 0·83), molinate sulfoxide, (R_f 0·55) and several unknown compounds with R_f values 0·86, 0·79, 0·72, 0·68 and at the origin. However, tobacco cells also formed unknown compounds with R_f values 0·76 and 0·03, and in rice cells, additional metabolites were found at R_f 0·95, 0·37 and 0·18. There are therefore significant differences in the metabolites produced by rice and tobacco cells. The aqueous layers (after acid

TABLE 4
Change in Amounts of [^{14}C]Molinate and Its Metabolites in Tobacco and Rice Cells and in Culture Medium with Incubation Time

	Amount found (%)									
	Tobacco[a]				*Rice*[a]					
		Metabolites[c]				*Metabolites*[c]			*In culture medium*[b]	
Incubation time (*h*)	*Molinate*	*MSO*	*MSO_2*	*Others*	*Molinate*	*MSO*	*MSO_2*	*Others*	*Tobacco*	*Rice*
3	23·0	1·6	3·0	72·4	18·6	2·3	3·8	75·3	81·8	83·9
6	11·8	1·5	3·0	83·7	14·0	1·8	3·5	80·7	85·7	83·0
12	10·8	2·0	3·0	84·2	9·3	2·0	3·4	85·3	86·5	83·2
24	9·7	1·3	1·7	87·3	10·3	2·6	2·8	84·3	81·0	85·9
48	6·8	1·4	2·8	89·0	6·5	1·3	2·0	90·2	84·2	85·9

[a] % of total radioactivity in cells.
[b] % of total radioactivity applied.
[c] MSO: molinate sulfoxide; MSO_2 : molinate sulfone.

analysis, fraction 7 in Fig. 1) contained only two spots, an unknown compound at R_f 0·72 and activity retained at the origin.

The amounts of molinate and the major metabolites, the sulfoxide and the sulfone of molinate, in cells of both species were determined (Table 4). The percentages of the radioactivity for the other metabolites in the cell and in the cell-culture fluid were determined separately and are shown in Table 4. Several papers[15–17] have reported that thiocarbamate herbicides must be metabolized in the plant to thiocarbamate sulfoxides to exhibit herbicidal activity, but this is disputed by Jablonkai and Hatzios.[18] Imai and Kuwatsuka[2] have proposed a metabolic pathway for molinate degradation in plants. They reported that molinate initially formed molinate sulfoxide, and this was then conjugated with glutathione or oxidized to molinate sulfone. In this study the differences in metabolism of molinate between rice and tobacco are minimal and do not appear to be a significant factor in selectivity. However, studies on metabolism in whole plants are required to clarify the role of metabolism further.

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